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# CD43 Is a Murine T Cell Costimulatory Receptor that Functions Independently of CD28

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## Summary

Costimulation mediated by the CD28 receptor has been shown to play an important role in the development of a vigorous T cell immune response. Nevertheless, CD28-deficient mice can mount effective T cell-dependent immune responses. These data suggest that other costimulatory molecules may play a role in T cell activation. In a search for other costimulatory receptors on T cells, we have characterized a monoclonal antibody (mAb) that can costimulate T cells in the absence of accessory cells. Similar to CD28 antibodies, this mAb, R2/60, was found to synergize with T cell receptor engagement in inducing proliferation. Independent ligation of CD3 and the ligand recognized by R2/60 results in T cell proliferation, suggesting that the two molecules do not have to colocalize to activate the R2/60 costimulatory pathway. R2/60 does not react with CD28, and furthermore, R2/60 costimulates in a CD28-independent fashion since the mAb costimulates T cells from the CD28-deficient mice as well as wild-type mice. Expression cloning of the R2/60 antigen identified the ligand as murine CD43. Together, these data demonstrate that CD43 can serve as a receptor on T cells that can provide CD28-independent costimulation.

The generation of a T cell response has been shown to depend on two independent signals (1). The first signal for activation is mediated by the interaction of the antigen-specific TCR with processed peptides displayed by APCs. This specific signal must be accompanied by a second costimulatory signal(s) delivered by the APC to the T cells through cell-cell interactions or cytokines. The lack of costimulation prevents proliferation through the inhibition of autocrine lymphokine production. Moreover, in the absence of a second signal, some T cells are induced into a state of nonresponsiveness often called "anergy" (2).

The nature of the costimulatory signal has recently been the subject of a large number of studies (3). CD28, which is expressed on a majority of T cells, is a receptor for the costimulatory molecules B7-1 and B7-2 (4). Binding of CD28, by either mAb or B7 family members, has been shown to costimulate antigen-specific T cell responses (5, 6), allogeneic mixed lymphocyte reactions (7, 8), and direct TCR-CD3

activation through antibody-mediated stimulation (9). In addition, the blocking of CD28/ligand interactions substantially, but not completely, inhibits these immune responses (3).

Recent studies, however, have shown that mice rendered CD28-deficient through mutations introduced by homologous recombination technology (CD28<sup>-/-</sup>), develop normal immune responses to viral infections in vivo, and reject allogeneic skin grafts (reference 10 and Green, J. M., C. B. Thompson, and J. A. Bluestone, unpublished data). The T cells from these mice can mount accessory cell-dependent responses to alloantigens, lectins, and anti-CD3 antibodies, although the response is decreased compared to normal control animals (10, 11). These data suggest that other cell surface molecules may provide costimulatory activity.

In this report, we have identified an additional costimulatory molecule on murine T cells distinct from CD28. This molecule is bound by a rat IgM mAb, R2/60, that was produced after immunization with murine intestinal epithelial lymphocytes (12). We find that the R2/60 mAb, in the absence of any other costimulatory interactions, can synergize with TCR engagement and induce T cell proliferation. Independent ligation of CD3 and the ligand recognized by R2/60, also leads to T cell proliferation, suggesting that

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R2/60 activates a costimulatory pathway that does not require colocalization with the TCR-CD3 complex. The mAb also costimulates T cells from CD28<sup>-/-</sup> mice as well as wild-type T cells, thus the R2/60-activated costimulatory pathway is independent of CD28. Using R2/60 to expression clone the cDNA that produces the R2/60 antigen, we found that R2/60 was specific for murine CD43 (leukosialin, Ly-48). Thus, CD43 can provide CD28-independent costimulation to T cells.

## Materials and Methods

**Mice.** C57BL/6 and BALB/c mice were purchased from the National Cancer Institute animal facility (Frederick, MD). CD28-deficient mice were previously described (10), and a colony of the CD28-deficient mice and wild-type (CD28<sup>+/+</sup>) control mice were maintained in a specific pathogen-free animal facility at the University of Chicago.

**Antibodies.** The procedure used to generate and isolate mAb R2/60 has been previously reported (12). Control Rat IgM was purchased from Zymed Laboratories (South San Francisco, CA). Hamster anti-murine CD28 (PV-1; reference 4) was a kind gift from Dr. Carl June (Naval Medical Research Institute, Bethesda, MD). All other flow cytometry reagents were purchased from PharMingen (San Diego, CA). Culture supernatant or ascites of anti-CD3 (145-2C11; reference 13), anti-heat-stable antigen (J11D; reference 14), anti-I-A<sup>b</sup> (25-9-3; reference 15), anti-I-A<sup>d</sup> (MKD6; reference 16), and anti-Fc receptor (2.4G2; reference 17) were prepared in the laboratory, purified as required, and the cell lines are available from American Type Culture Collection (Rockville, MD). Alkaline phosphatase-conjugated goat anti-rat Ig (Jackson Laboratories, Inc., West Grove, PA) was used to develop the Western blot stained with R2/60. Anti-CD43 (S7, rat IgG2a) was generously provided by J. Frelinger (Cancer Center, Immunology Division, University of Rochester, NY). Fab' fragments of the S7 antibody were made by standard methods and purity was confirmed by SDS-PAGE (data not shown).

**Flow Cytometry.** Two-color staining with directly conjugated FITC or biotinylated antibodies was done in the presence of anti-FcR (2.4G2) culture supernatant for 30 min. After washing, biotin reagents were developed with phycoerythrin-conjugated streptavidin (Southern Biotechnology Associates, Birmingham, AL). Dead cells were excluded from the analysis by DNA staining with propidium iodide, and the cells were analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA).

**T Cell Purification and Proliferation Assays.** Lymph node T cells were purified as previously described (18). Briefly, total lymph node cells were passed through a nylon wool column, and nonadherent cells were collected. T cells were further purified by complement-mediated cytotoxic treatment with a mixture of anti-Ia (25-9-3 for I-A<sup>b</sup>, and MKD6 for I-A<sup>d</sup>) and anti-heat-stable antigen (J11D) antibodies. Dead cells were removed by centrifugation through Ficoll-Hypaque. T cell purity was analyzed by flow cytometry and Con A stimulation. Purified T cells were consistently >97% CD3 positive. In experiments where the Con A response of the purified T cells was tested, the response of the purified T cells was <2% of the response seen when irradiated Ia<sup>+</sup> APC were added (data not shown). For mixed lymphocyte cultures, total lymph node cells ( $4 \times 10^5$ ) or purified T cells ( $2 \times 10^5$ ) from wild-type or CD28<sup>-/-</sup> mice were isolated and cocultured with  $10^5$  irradiated BALB/c splenocytes.

In some experiments, antibodies were diluted in PBS and im-

mobilized to individual wells of 96-well flat bottom microtiter plates in a final volume of 200  $\mu$ l. The plates were incubated at 37°C overnight. Before use, the plates were washed twice with unsupplemented DMEM media (Gibco Laboratories, Grand Island, NY), and blocked for 15 min with DMEM media supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.29 mM L-glutamine, nonessential amino acids, 10 mM Hepes, and  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol.

Polystyrene beads ( $7 \times 10^7$ ) from Polysciences, Inc. (Warrington, PA) were independently coated with either 145-2C11, R2/60, or control rat IgM by incubating 100  $\mu$ g of purified mAb in 1 ml of 500 mM sodium carbonate buffer at pH 8.5. After an overnight incubation at room temperature, the beads were washed four times to remove unbound antibody and were then used in experiments as noted.

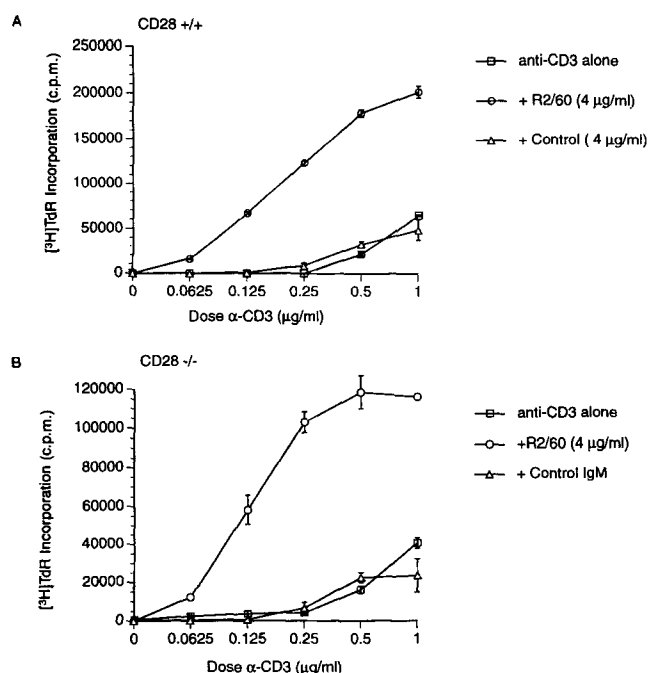
Purified T cells were cultured at  $2 \times 10^5$  cells per well. Except when noted, experiments were pulsed with 1  $\mu$ Ci [<sup>3</sup>H]thymidine at 48 h after initiation of the culture, and harvested 16 h later. Plates were harvested on a 96-well harvester (Filtermate 196; Packard Instrument Co., Meriden, CT), and counts were read on a microplate scintillation counter (Topcount; Packard Instrument Co.). All data points were performed in triplicate, and data were presented as mean  $\pm$  SD.

**Western Analysis.** Cells were washed twice in PBS and then lysed at a final concentration of  $10^8$  cells per ml in 0.5% NP-40 lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 25  $\mu$ M *p*-nitrophenyl *p*'-guanidino-benzoate, 10 mM NaF, and 10 mM iodoacetamide). 50  $\mu$ l of each lysate was separated on a 10% discontinuous Laemmli SDS-PAGE gel. The protein was transferred to nitrocellulose by electrotransfer apparatus. Nonspecific binding was blocked by preincubating the blot overnight in PBS containing 5% dry milk. After washing, the blot was incubated with 10% R2/60 culture supernatant in TBST (10 mM Tris, 150 mM NaCl, and 0.05% Tween 20) for 2 h, washed extensively, and developed with alkaline phosphatase-conjugated goat anti-rat IgG (Jackson Laboratories). Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate substrates were added.

**Expression Cloning.** The R2/60 antigen was cloned using the method of Seed and Aruffo (19). Briefly, a cDNA library in the eukaryotic expression pCDNA1 (Invitrogen, San Diego, CA), generously provided by Dr. L. Matis (Alexion Pharmaceuticals, New Haven, CT), was constructed from LPS-activated murine splenocytes and transiently transfected into COS-7 cells by DEAE-dextran. Cells expressing the R2/60 antigen were enriched by panning over R2/60 antibody-coated plates. The episomal DNA was rescued by the method of Hirt (20) and subsequent rounds of transfection and panning were performed. After three rounds, individual clones were retransfected into COS-7 cells and screened by FACS® (Becton Dickinson & Co) analysis for R2/60 surface staining. Positive clones were isolated and sequence analysis was performed by the dideoxy method.

## Results

**The R2/60 mAb Provides Costimulation in the Absence of Accessory Cells.** In our search for novel costimulatory receptors on T cells, different mAbs were examined for their ability to augment T cell proliferation in the absence of accessory cells. One rat IgM monoclonal antibody, R2/60, activated purified T cells when coimmobilized with submitogenic doses of anti-CD3 (Fig. 1 A). The R2/60 mAb increased the proliferative response at all doses of anti-CD3, but did not



**Figure 1.** The R2/60 mAb can costimulate anti-CD3-mediated T cell proliferation in the absence of APC and independent of CD28 stimulation. (A) Anti-CD3 (145-2C11) was coimmobilized with the R2/60 antibody at the indicated concentrations. Purified T cells were plated at  $2 \times 10^5$  cells per well, and pulsed with [ $^3$ H]thymidine at 48 h after the initiation of the culture, and harvested 16–18 h later. The data presented are representative of more than 15 independent experiments. (B) T cells from CD28-deficient mice were cultured as described above. The data presented are representative of three independent experiments.  $\square$ —, anti-CD3 alone;  $\circ$ —, + R2/60 (4  $\mu$ g/ml);  $\triangle$ —, + control IgM (4  $\mu$ g/ml).

induce proliferation in the absence of coimmobilized anti-CD3. Thus, R2/60 cross-linking, in the absence of APC, can provide a “second signal” necessary to induce T cell proliferation in response to suboptimal anti-CD3 stimulation.

**The Antigen Recognized by R2/60 Is Not CD28.** To test the relationship of the R2/60 ligand to CD28, its expression was analyzed in both wild-type and CD28-deficient mice. In contrast to CD28, R2/60 was expressed on a wide variety of cells (data not shown). Furthermore, the mAb was equally reactive with cells isolated from CD28-deficient mice (Fig. 2). Thus, these data excluded CD28 as a candidate for the R2/60 antigen.

**R2/60-mediated Costimulation is CD28 Independent.** Although R2/60 does not react with CD28, many of the features of the R2/60-mediated costimulation were functionally similar to CD28 costimulation. Thus, it was possible that signaling through R2/60 ligation was linked to CD28 ligation and signaling. To address this possibility, the ability of R2/60 to augment the proliferation of T cells from CD28-deficient mice was studied. Purified T cells from wild-type and CD28 $^{-/-}$  mice were cultured with submitogenic doses of anti-CD3 alone or in the presence of coimmobilized R2/60 mAb or control mAb (Fig. 1 B). At all concentrations tested, R2/60 cross-linking enhanced the proliferation of the

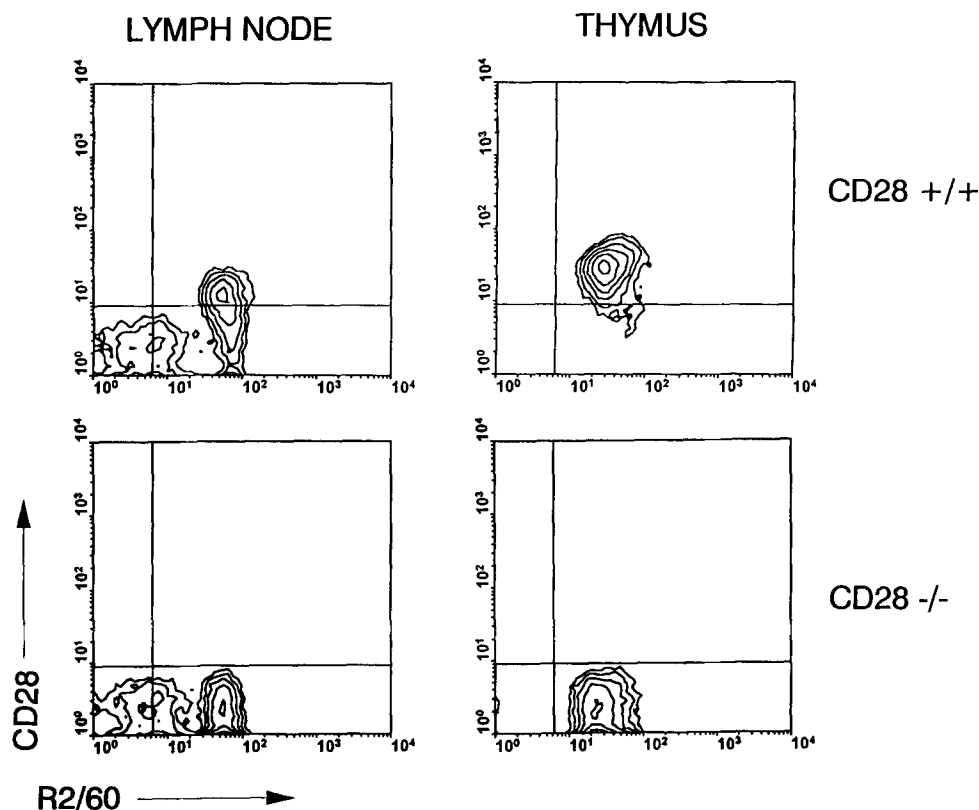
CD28 $^{-/-}$  T cells equivalently to wild-type T cells. Thus, R2/60-mediated costimulatory signals do not require CD28 ligation or expression, and therefore can function independently of CD28.

**R2/60-mediated Costimulation Is Not Dependent on Colocalization with the TCR.** The increased proliferation that occurs with the coimmobilization of R2/60 and anti-CD3 may be caused by an independent costimulatory signal delivered through the molecule recognized by R2/60, or alternatively, it may be caused by an increased adhesive interaction between TCR and anti-CD3. To distinguish between these possibilities, the R2/60 molecule and CD3 were independently ligated on purified T cells using polystyrene beads coated separately with either R2/60 mAb or anti-CD3 (Fig. 3). The R2/60-coated beads augmented the T cell proliferative response induced by anti-CD3-coated beads, but had no effect alone. Thus, the proliferative response was the result of a separate costimulatory signal, and not the result of increased adhesion of the T cells to the immobilized anti-CD3.

**The Antigen Recognized by the R2/60 mAb is the Murine Homologue of CD43.** To begin to identify the antigen recognized by R2/60, both flow cytometry and Western analyses of whole-cell lysates from various cell types were studied. Flow cytometric analysis demonstrated that the molecule recognized by R2/60 mAb is expressed on a large number of cell types including T cells, B cells, thymocytes, monocytes, and fibroblasts (Fig. 2 and data not shown). Further biochemical studies demonstrated that R2/60 reacts with a protein of 115 kD apparent mol mass (Fig. 4). A second protein of 105 kD mol mass was detected by the R2/60 mAb, and was expressed at varying levels in different populations. The apparent molecular weight of these proteins does not change when analyzed under reducing conditions (data not shown), indicating that the surface antigen detected by R2/60 is a monomeric protein with few, if any, intramolecular disulfide bonds.

To identify the surface receptor recognized by the R2/60 mAb, we undertook an expression cloning approach. COS-7 cells were transfected with a cDNA library from LPS-stimulated splenocytes. Cells that contained a plasmid coding for a protein recognized by the R2/60 antibody were enriched by panning over plates coated with the antibody. The episomal DNA was rescued and amplified in bacteria and then retransfected into COS. After repeating this for three cycles, individual clones were isolated and screened for their ability to direct surface expression of the R2/60 antigen (Fig. 5 A). Two independent clones were isolated and further characterized. Sequence analysis revealed the clones to be identical to the published sequence for the murine homologue of CD43 (leukosialin, Ly-48; reference 21). Retransfection of one of the clones into COS-7 cells demonstrated that both R2/60 and a commercially available anti-CD43 antibody recognized the transfected cells (Fig. 5 B and reference 22). Thus, the identity of the surface receptor recognized by the R2/60 mAb is murine CD43.

**Costimulation Mediated through CD43 Requires Cross-linking.** We next examined whether the augmented proliferation observed by the addition of anti-CD43 antibody was



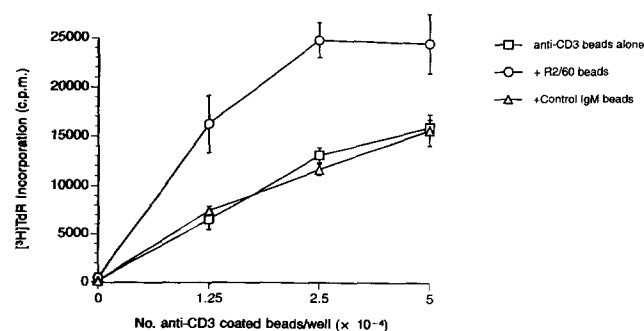
**Figure 2.** The R2/60-specific molecule is expressed on lymphocytes and thymocytes from CD28-deficient mice. Lymph node cells and thymocytes were stained with FITC-coupled R2/60 and PE-conjugated anti-CD28.

dependent on cross-linking of CD43 on the cell surface. One approach is to use monovalent Fab' fragments of an anti-CD43 antibody. Since R2/60 is an IgM, another anti-CD43 antibody, S7, which is a rat IgG2a, was used for these experiments. As seen in Fig. 6, the addition of S7 augmented the proliferative response of alloantigen-activated T cells from wild-type and CD28-deficient mice. When Fab' fragments from the S7 antibody were included, the proliferative response

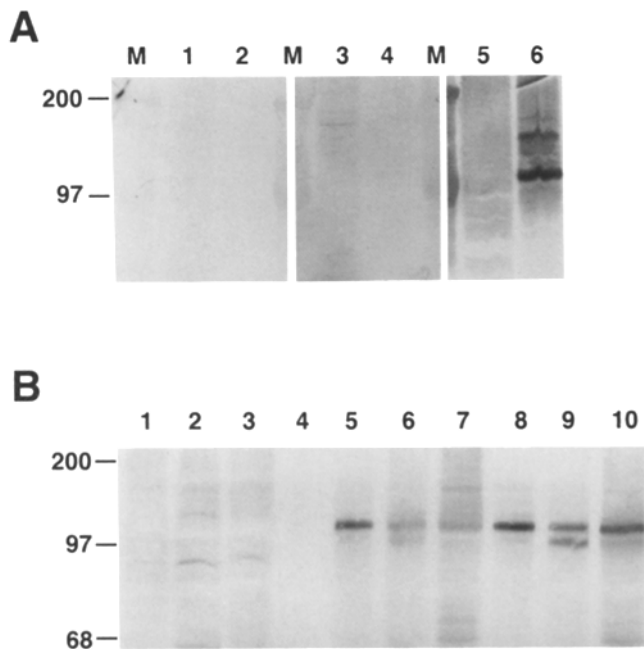
was not enhanced. This suggests that cross-linking of CD43 may be important since the addition of secondary antibody directed against the Fab' fragments reconstituted the costimulatory activity of the S7 antibody.

## Discussion

The two-signal hypothesis for T cell activation has suggested that, in addition to a specific antigenic signal, T cell proliferation requires costimulatory signals provided by the accessory cell. Many studies have demonstrated that CD28 on T cells is a costimulatory receptor for activation, although T cells from CD28-deficient mice are still able to produce immune responses to pathogens (10). In addition, CD28-deficient T cells can be activated in vitro and in vivo, albeit at a lower magnitude than normal T cells (10, 11). One potential explanation for these results is the existence of other costimulatory receptors on T cells. In this study, we describe the cloning and characterization of an alternative costimulatory receptor on T cells. We have shown that the antibody R2/60 is specific for murine CD43. Ligation of CD43 on murine T cells with this mAb synergizes with submitogenic doses of anti-CD3 mAb to induce the proliferation of T cells in the absence of accessory cells or CD28 signaling. Similar to our studies in the mouse, several groups have found that CD43 ligation can enhance the proliferation of human T cells (23, 24). Thus, CD43 may regulate T cell activation through a costimulatory pathway that is independent of CD28.



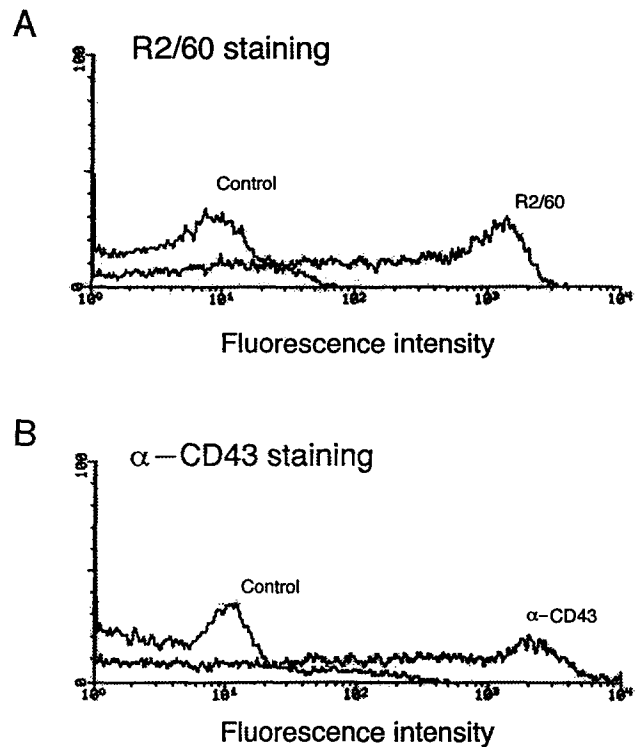
**Figure 3.** R2/60-mediated costimulation does not require colocalization with the T cell receptor. Polystyrene beads were coated with either anti-CD3 (2C11), R2/60, or control rat IgM. The anti-CD3-coated beads were added as indicated, and the R2/60 and rat IgM-coated beads were added at  $2.5 \times 10^4$  beads per well. Cultures were pulsed and harvested as in Fig. 1. The data presented are representative of three experiments. □, anti-CD3 beads alone; ○, + R2/60 beads; △, + control IgM beads.



**Figure 4.** Immunoblotting with the R2/60 mAb reveals proteins of 105 kD and 115 kD apparent molecular weight. (A) COS cells (lanes 1, 3, and 5) and total lymph node cells (lanes 2, 4, and 6) were blotted with R2/60 (lanes 5 and 6), control rat IgM, 7D4 ( $\alpha$ -IL-2R; lanes 3 and 4) and alkaline phosphatase-conjugated goat anti-rat Ig (all lanes). (B) Total cell lysates of (lane 1) COS cells, (lane 2) CHO cells transfected with murine B7-1, (lane 3) CHO cells transfected with murine CD28, (lane 4) blank, (lane 5) total lymph node cells, (lane 6) T-depleted splenocytes, (lane 7) cell from lane 6 after LPS activation, (lane 8) purified lymph node T cells, (lane 9) B-depleted splenocytes, and (lane 10) cells from lane 9 after Con A stimulation were blotted with R2/60 followed by alkaline phosphatase-conjugated goat anti-rat Ig.

CD43 is a large mucin-like protein containing one N-linked carbohydrate and 70–90 O-linked oligosaccharides, and its apparent molecular weight varies between populations because of differential glycosylation. The apparent molecular weights characterized in Fig. 3 are similar to those previously reported for CD43 (25). Thus, these data suggest that the R2/60 antibody reacts with at least two alternatively glycosylated forms of CD43.

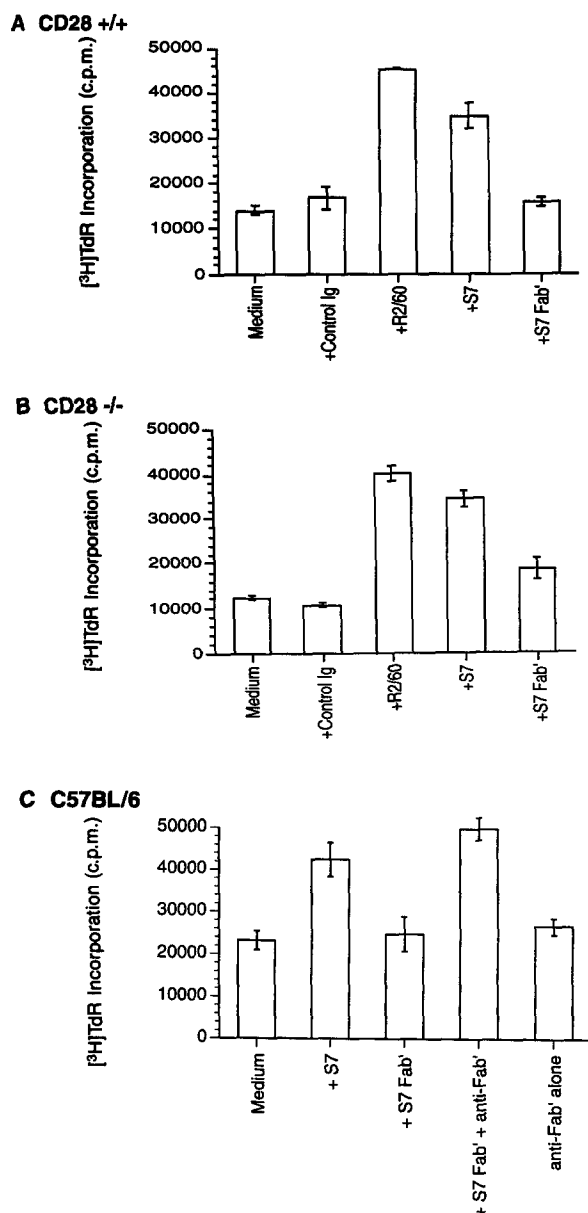
The nature of the mechanism by which CD43 increases T cell proliferation is unknown. There are at least two possible hypotheses that can be envisioned. First, R2/60 may mimic a classical signaling mechanism by which CD43 is ligated normally through interactions with ligand(s) leading to a signal transduced through its cytoplasmic tail, which results in the production of autocrine lymphokines. A role for signal transduction is supported by the need for cross-linking to observe an augmented proliferative response. It is important to note that the protein sequence of the intracellular region of murine CD43 gene is highly homologous with rat CD43 (90%) and human CD43 (72%) (21). This strong evolutionary conservation suggest important functional activity for this region. In addition, several groups have shown that CD43 in T lymphocytes (26) and monocytes (27) is con-



**Figure 5.** The R2/60 mAb identifies the murine homologue of CD43. COS-7 cells transiently transfected with the plasmid DNA isolated through expression cloning were stained with (A) R2/60 or (B) anti-CD43 (S7; PharMingen).

stitutively phosphorylated and hyperphosphorylated after activation. While the cytoplasmic CD43 sequence has no catalytic regions itself, these data suggest that it may associate with other protein kinases or signal transduction molecules. Other groups have shown a possible association of CD43 signaling with protein kinase C, but the nature of the association is not yet clear (28). If CD43 functions by initiating a signal transduction cascade, it will be important to identify its ligand(s). This ligand(s) may be expressed on “professional APC” such as dendritic cells, macrophages, or activated B cells. Or alternatively, the ligand(s) may be expressed on cell types involved in cell homing or localized T cell activation as in the germinal centers. Finally, it is possible that the ligand exists on the T cells, similar to what has been postulated for CD45 (29). Previously, it has been found that intracellular adhesion molecule 1 can bind CD43 and therefore, may be a potential ligand (30). Further studies are necessary to determine the role of intracellular adhesion molecule 1 or other potential ligands in the regulation of T cell responses by CD43.

A second, nonclassical, mechanism may be proposed for CD43 function based on its biochemical features. Since CD43 is a highly glycosylated, negatively charged molecule that is expressed at high levels on many lymphocytes, it is possible that cell-cell contact is impeded by its expression. This repulsion may provide a physical restraint on the ability of APC to interact with T cells and to provide appropriate activation



**Figure 6.** Anti-CD43 antibodies require cross-linking to augment the proliferative response of alloantigen-activated T cells. Lymph node cells from (A) CD28<sup>+/+</sup> or (B) CD28<sup>-/-</sup> mice were isolated and stimulated with irradiated allogeneic splenocytes in the presence of medium alone, control Ig (4  $\mu$ g/ml), R2/60 (4  $\mu$ g/ml), S7 (4  $\mu$ g/ml), or Fab' fragments of S7 (50  $\mu$ g/ml), and proliferation determined by tritiated thymidine incorporation for the final 16 h of a 64-h culture. (C) Purified T cells from C57BL/6 mice were cultured as above in the presence of medium alone, S7 (0.5  $\mu$ g/ml), S7 Fab' (50  $\mu$ g/ml) alone, or with the inclusion of a goat anti-rat Ig (20  $\mu$ g/ml) to cross-link the bound Fab' fragments.

signals. CD43 has been found to be proteolytically cleaved from the surface of activated lymphocytes and granulocytes (31), especially when CD43 was directly cross-linked. In addition, a soluble galactoglycoprotein (Galgp), found in high concentrations in human serum, has been found to have exact sequence identity with the extracellular region of CD43 (32). Thus, a second possible mechanism for CD43-mediated costimulation of T cells may be that shedding of the extracellular domain may enhance intercellular interactions, and therefore, critical receptor/ligand interactions. This mechanism does not exclude the need for an intracellular signal to be generated by CD43. This hypothesis is supported by recent reports demonstrating that transfection of HeLa cells with CD43 actually reduces the ability of these cells to bind to T cells (33), and that targeted disruption of the CD43 gene in the CEM T cell line increases cellular adhesion (34). However, we and others have found an increased level of expression of CD43 when T cells are activated with Con A or CD3 (reference 35 and data not shown). Therefore, the proteolytic cleavage of CD43 from the surface of lymphocytes may not be a necessary consequence of lymphocyte activation. Further studies will be necessary to distinguish the two potential mechanisms of CD43-mediated costimulation.

The exact role of CD43 in the T cell immune response remains to be determined. While we have found that anti-CD43 can costimulate T cell responses, it is not known under what circumstances CD43 ligation is necessary for T cell activation. Fab' fragments of an anti-CD43 antibody failed to inhibit alloantigen activation of lymph node cells; however, there are multiple possible explanations for this result. It is possible that the antibody recognizes an epitope on CD43 different from the natural ligand, and therefore may not be able to sterically hinder receptor-ligand interactions. This would be especially likely if the ligand for CD43 is a c-type lectin that interacts with the extensive terminal carbohydrate modifications of CD43. In addition, the difference in affinity of the natural ligand as compared to the Fab' fragments for CD43 may be too great to allow for effective competition. Although flow cytometry staining with S7 was blocked by Fab' fragments, the Fab' bound with significantly lower affinity than whole antibody (data not shown). Future studies using a variety of genetic and molecular approaches will provide important insights into both the functional role of CD43 in T cell costimulation and the mechanism by which CD43 mediates its function.

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